UNIVERSITY OF WISCONSIN-MADISON
Research Administration-Financial

Telephone 608-262-3822
FAX 608-262-5111

750 University Avenue
Madison, Wisconsin 53706-1490

[redacted]

Reply to Attn. of
Proposal #64511

Consortium for Plant Biotech.
Research Inc.
1220 Potter Drive
Suite 130D
West Lafayette, IN 47906

Ladies and Gentlemen:

Submitted herewith, in behalf of the Board of Regents of the University of Wisconsin System, is a revised application in the amount of $112,000 under the direction of Dr. S. Austin-Phillips, Dr. T. German and Dr. R. R. Burgess all of the Biotechnology Center.

The application for subject proposal was originally submitted under letter of [redacted]

This revised application has been administratively approved and is submitted for your consideration. Please keep our office advised as developments occur with regard to this application.

Please use the University's above-referenced proposal number in any future correspondence. Questions, or requests for further information, should be directed to Cindy Marshall at (608) 262-9727.

Sincerely,

Cheryl E. Gest

Cheryl E. Gest
Administrative Officer

CEG:CM:pf
Enclosure
cc: Asst. Dean J. Knickmeyer
Dr. S. Austin-Phillips
Dr. T. German
Dr. R. R. Burgess
Dir., Biotech. Ctr.

REVISED SCOPE OF WORK

TRANSGENIC ALFALFA AS AN ALTERNATIVE ECONOMICAL SOURCE OF LIGNOCELLULOSIC DEGRADING ENZYMES FOR USE IN BIOMASS CONVERSION.

S. Austin-Phillips, T.L. German & R.R. Burgess.

1. SUMMARY.

Lignocellulosics offer tremendous potential for the production of fuel and as a chemical feedstock. This can be an economically-viable alternative to the use of fossil fuels if sugars can be efficiently and completely recovered from hydrolysis of the polysaccharide components of biomass, hemicellulose and cellulose. Acid hydrolysis of cellulose is a relatively cheap process but yields are low due to chemical alteration of sugars to non-fermentable, growth inhibitory compounds. Acid-based processes also generate a difficult and environmentally unfriendly waste stream. Enzymatic hydrolysis of cellulose gives a single, pure product, glucose, but is more costly. Enzyme production is a very large single component cost in the biomass conversion process. Thus, a major limiting factor in realizing the potential of plant biomass is the cost and availability of large amounts of cellulose-degrading enzymes. This project will determine if cellulases can be produced at a lower cost using plants as a bioreactor as compared to fermentation processes. The long term goals of the research are to develop genetically engineered alfalfa that produces high levels of enzymes (cellulases) and to develop the technology needed to extract these enzymes from alfalfa juice.

2. RESEARCH GOALS.

The funding for this project was reduced by 50% which will significantly impact the amount of research that can be done in a two year period. The overall research objectives and specific goals will essentially remain the same, but the actual experimentation will be scaled down.

The specifics of the research are as follows:

A. Construct plant expression vectors carrying genes coding for cellulases.

In our original proposal we intended to obtain cellulase coding sequences from collaborators at NREL for three cellulases; (1) Cellobiohydrolase I (CBHI) from *Trichoderma reesei*, (2) a ß-1,4-endoglucanase gene originally isolated from a thermotolerant bacterium, *Acidothermus cellulolyticus* (E1), and (3) a ß-1,4-exoglucanase gene originally isolated from a different thermotolerant bacterium, *Thermomonospora fusca* (E3),(see attached letter #1). It now appears that two of these clones will not be available in the near future for our use (see letter #2). We hopefully will still be able to receive the clone for CBH-1 from NREL. However, Dr. P. Wilson from Cornell University has agreed to make available clones for two cellulases from *T. fusca*, E2 and E3, (see letter #3). Thus we will have cellulase clones for this project. Due to a reduction in funding we will probably chose one of the enzymes for our initial study instead of trying to work with all three at the same time.

Initially the cellulase coding sequences will be inserted into expression vectors we have used in the past to express foreign proteins in alfalfa. Briefly, these vectors will consist of the binary plant transformation vector pCGN1578 (McBride and Summerfelt, 1990) into which was inserted a plant expression cassette containing the coding sequence of our gene of interest. The expression cassette contains the "Mac" promoter (Comai *et al.*, 1990) and the mannopine synthase

transcription terminator (McBride and Summerfelt, 1990). Based on primary observations it may be necessary to modify the expression vectors to maximize foreign protein production and accumulation. We will remove the microbial signal sequence from the gene and determine if this results in cytoplasmic accumulation. This may be desirable since there is no substrate for the enzyme in the cytoplasm. If this does not result in cellulase expression we will use other constructs we have on hand which will allow us to compare the effect on accumulation of localization of the protein to the ER (Wandelt *et al.*, 1992), vacuole (Bednarek and Raikhel, 1991) and apoplast (Mason *et al.*, 1988). This may be important not only to maximize protein accumulation, but also to avoid any deleterious effects of the cellulases on the host plant's metabolism.

2. Transform alfalfa with genes coding for cellulases.

We will introduce the chimeric cellulase genes into alfalfa using procedures that are well established in our laboratory. In response to one of the reviewers comment the actual procedure is given below. This transformation method was defined after testing several modifications of the basic explant cocultivation system (Horsch *et al.*, 1985) as ways to optimize the transformation procedure. These included pretreatment of the tissue, longer cocultivation times and the use of different levels of the antibiotic kanamycin monosulphate to select for transformed tissue. The optimized procedure is as follows. New-growth trifoliates are taken from RSY27 plants maintained in a growth room (conditions as described above) and sterilized using alcohol and bleach washes (30s in 70% alcohol, 90s in 20% hypochlorite + 0.1% SDS, followed by three rinses in sterile distilled water). Leaf edges are cut on moist filter paper and tissue dropped into liquid SH-II medium (Bingham *et al.*, 1975). When sufficient explants have been taken, they are moved to a suspension of *Agrobacterium* cells (containing the engineered plasmid) from an overnight culture grown in liquid YEP selection medium. Cell density was adjusted to fall between 0.6-0.8 at A_{660}. After 30 minutes inoculation, the explants are gently blotted on filter paper and placed on B5H medium (Brown and Atanassov, 1985) for 4 days. They are then rinsed twice in sterile water and cultured on B5H for a further 4 days. At the end of this period, they are rinsed three times and transferred to B5H containing 25 mg L^{-1} kanamycin and 250 mg L^{-1} carbenicillin. Plates are maintained at 24°C, 16h photoperiod and light intensity of 60-80 $\mu E\ m^{-2}\ s^{-1}$. Explant-derived calli (and occasionally embryoids) which formed within 3 weeks on this medium are moved to B5H with antibiotics but without hormones to allow for further embryoid production and development of existing embryoids. After 3-4 weeks, embryos were transferred to MS medium (Murashige and Skoog, 1962) plus the two antibiotics to allow for development into plantlets. Callus forms on untreated explants in the presence of 25 mg L^{-1} kanamycin but embryos are never produced. Each explant piece can give rise to multiple (up to 40) embryos. Plantlets are rooted on MS medium lacking antibiotics. For evaluation purposes only, one rooted plant per explant piece is assessed, thus assuring that each is the result of an independent transformation event.

The transformed plants will be analyzed for levels of enzyme production and grown to maturity to determine if there are any deleterious effects on growth and development.

We have already produced alfalfa expressing enzymes with current or potential industrial usage and are currently evaluating several methods for the efficient and economic recovery of the expressed proteins. Thus we are in an excellent position to determine if this approach is a viable alternative to fermentation for producing large amounts of enzymes needed in biomass conversion.

3. TIME FRAME

It is difficult to predict the time required to determine if cellulases can be expressed in plants. We estimate this will be in the range of 12 - 24 months. Maximizing expression and recovery of the enzymes will take a further 12 - 24 months.

References

Bednarek, S.Y. and N. Raikhel, 1991. The barley lectin carboxy-terminal peptide is a vacuolar protein sorting determinant in plants. The Plant Cell 3:1195-1206.

Bingham, E.T., L.V. Hurley, D.M. Kaatz & J.W. Saunders, 1975. Breeding alfalfa which regenerates from callus tissue in culture. Crop Sci 15: 719-721.

Brown, D.C. & A. Atanassov, 1985. Role of genetic background in somatic embryogenesis in *Medicago*. Plant Cell Tissue Organ Culture 4: 107-114.

Horsch, R.B., J.E. Fry, N.L. Hoffman, D. Eicholtz, S.G. Rogers & R.T. Farley, 1985. A simple and general method for transferring genes into plants. Science 227: 1229-1231.

Comai, L., P. Moran, & D. Maslyar, 1990. Novel and useful properties of a chimeric plant promoter combining CaMV 35S and MAS elements. Pl. Mol. Biol. 15: 373-381.

Mason, H.S., F.D. Guerrero, J.S. Boyer & J.E. Mullet, 1988. Proteins homologous to leaf glycoproteins are abundant in stems of dark-grown soy bean seedlings. Analysis of proteins and cDNAs. Plant Mol Biol 11: 845-856.

McBride, K.E. & K.R. Summerfeldt, 1990. Improved binary vectors for *Agrobacterium* mediated plant transformation. Plant Mol Biol 14: 269-276.

Murashige, T. & F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473-497.

Wandelt, C., M.R.I. Khan, S. Craig, H.E. Schroeder, D. Spencer, & T.J.V. Higgins, 1992. Vicilin with carboxy-terminal KDEL is retained in the endoplasmic reticulum and accumulates to high levels in the leaves of transgenic plants. Plant J. 2: 181-192.

60 WORD SUMMARY

This project will determine if cellulases for use in biomass conversion can be produced in transgenic alfalfa The long term goals of the research are to develop genetically engineered alfalfa that produces high levels of cellulases and to develop the technology needed to extract these enzymes from alfalfa juice.